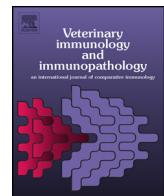




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Short communication

Development and characterization of mouse monoclonal antibodies reactive with chicken TL1A



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ABSTRACT

Tumor necrosis factor-like ligand 1A (TL1A) is a type II transmembrane protein predominantly expressed by endothelial cells that promotes the expansion of activated T cells and regulatory T cells, modulates inflammation, and regulates the production of a wide variety of T cell cytokines. However, there have not been any mAbs which specifically detect chTL1A and define its biochemical and immunological properties. So in this study, two mouse monoclonal antibodies (mAbs) which specifically detect chicken TL1A (chTL1A) were developed and characterized. Both mAbs identified a 32 kDa *Escherichia coli*-derived, poly-histidine-tagged fusion protein by Western blot analysis. The mAbs identified TL1A-secreting cells in the chicken thymus, cecal tonsil, and bursa of Fabricius by immunocytochemistry, and were used to measure serum TL1A levels in normal and necrotic enteritis (NE)-afflicted chickens by antigen capture ELISA. These mAbs inhibited chTL1A-induced spleen lymphocyte proliferation, nitric oxide production by chicken macrophage cells (HD11), and blocked the cytotoxic effect of chTL1A against lymphoblastoid chicken B tumor cells (LSCC-RP9). These new mAbs that detect chTL1A will be important immune reagents for basic and applied research in poultry immunology.

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1. Introduction

Tumor necrosis factor-like ligand 1A (TL1A) known as tumor necrosis factor superfamily 15 (TNFSF15) is a type II transmembrane protein predominantly expressed by

endothelial cells. In mammals, TL1A promotes the expansion of activated T cells and regulatory T cells, modulates inflammation, and regulates the production of a wide variety of T cell cytokines (Hou et al., 2005; Haritunians et al., 2010; Jones et al., 2011; Cohavy et al., 2011). *In vivo* treatment with TL1A-blocking antibodies (Abs) has revealed a specific role for TL1A in enhancing T cell proliferation at the sites of tissue inflammation in animal models of autoimmunity (Qin, 2011). TL1A expression is highly regulated, being virtually undetectable in normal tissues but highly induced upon stimulation with TNF- α , IL-1 α , Toll-like receptor ligands, such as lipopolysaccharide (LPS), and Fc-receptor

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cross-linking of macrophages and dendritic cells (Migone et al., 2002; Prehn et al., 2007; Meylan et al., 2008).

TNF-like activity has been detected in the supernatant of chicken macrophage culture medium although the molecular cloning of TNF- α has been unsuccessful in avian species (Rautenschlein et al., 1999). ChTL1A is a homolog of human TL1A and functions as an inflammatory cytokine like mammalian TNF- α (Park et al., 2007). LPS-induced plasma chTL1A level is reduced by rabbit anti-chTL1A pAb (Takimoto et al., 2005, 2008); however, there have not been any mAbs which specifically detect chTL1A. Therefore, the current study was conducted to develop mouse monoclonal antibodies (mAbs) against chTL1A to further define its biochemical and immunological properties.

2. Materials and methods

2.1. Expression and purification of recombinant chTL1A (rchTL1A)

The full-length TL1A mRNA was isolated by RT-PCR as described (Min and Lillehoj (2004)) using primers based on the published sequence (Takimoto et al., 2005) and the resultant full-length chTL1A cDNA was cloned into the pET32a bacterial expression vector (Novagen, Madison, WI) incorporating an NH₂-terminal poly-histidine epitope tag (Song et al., 1997). The recombinant chTL1A/pET32a gene construct was transformed into *Escherichia coli* BL21(DE3) and the cells were induced in 2X TY liquid culture medium (16 g Bacto tryptone, 10 g Yeast extract, 5 g NaCl/L) with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside in mid log phase ($OD_{600} = 0.4$) at 37 °C for 3 h. The recombinant chTL1A/pET32a (rchTL1A) fusion protein was purified by Ni²⁺-NTA His-bind resin column chromatography (Novagen) as described (Hong et al., 2008). The concentration of the purified rchTL1A protein was determined by the Bradford assay and protein purity was confirmed by SDS-PAGE and Western blotting (below).

2.2. ChTL1A mAb production and validation of antigen specificity by ELISA

All animal protocols were approved by the Beltsville Agricultural Research Center Institutional Animal Care and Use Committee. BALB/c mice (National Cancer Institute, Frederick, MD) were immunized biweekly by intraperitoneal and subcutaneous injections with 50 µg of purified rchTL1A protein in Freund's adjuvant (Sigma, St. Louis, MO) as described (Lee et al., 2011a,b, 2012). Booster injections were given intravenously with 25 µg of chTL1A without adjuvant at 3 days prior to cell fusion. Splenic lymphocytes were fused with mouse SP2/0 cells (ATCC) and hybridomas were selected in RPMI 1640 medium supplemented with hypoxanthine, aminopterin, and thymidine (all from Sigma). Hybridomas secreting chTL1A mAbs were selected by enzyme-linked immunosorbent assay (ELISA) as described (Yun et al., 2000; Min et al., 2002).

Briefly, 96-well microtiter plates were coated overnight at 4 °C with 1.0 µg/well of purified rchTL1A protein. As a negative control, an identical set of plates was coated with 1.0 µg/well of purified recombinant chicken interleukin

8 (rchIL8)/pET32a protein (Wu et al., 2008) instead of rchTL1A. The plates were blocked with PBS containing 1.0% BSA and washed with PBS, pH 7.2, containing 0.05% Tween 20 (PBS-T). Undiluted hybridoma culture supernatants (100 µl/well) were added, incubated with agitation at room temperature for 1 h, and washed with PBS-T. An irrelevant mAb detecting rchIL8 was used as a negative control. Bound mAbs were detected with horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary Ab (1/1000 dilution), 3,3',5,5'-tetramethylbenzidine substrate, and H₂O₂ (all from Sigma). Optical density at 450 nm (OD_{450}) was determined with a microplate reader (Bio-Rad, Richmond, CA). Hybridoma supernatants containing mAbs that reacted with rchTL1A, but not with rchIL8 protein, were selected for limiting dilution and further characterizations. Immunoglobulin isotypes were determined using mouse mAb isotyping kit (Sigma) according to the manufacturer's instructions. In this study, TL1A and IL8 mAbs, which are all IgG1 isotypes, were used.

2.3. Western blot analysis

Purified rchTL1A, rchIL8, or the rchTL1A protein, treated with proteinase K (Sigma) at 100 ng/ml for 12 h, was mixed with an equal volume of 0.125 M Tris-HCl, pH 6.8, 4.0% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.004% bromophenol blue, and heated at 95 °C for 5 min. Each protein (2 µg/lane) was resolved on a 15% SDS-polyacrylamide gel and electroblotted onto nitrocellulose (Immobilon-P, Millipore, Bedford, MA). The membrane was blocked with 1X PBS, pH 7.2, containing 5.0% nonfat dry milk, washed with 1X PBS with 0.1% tween 20 (PBS-T), and incubated with mAb chTL1A-1, -2, or their mixture mAb (1.0 µg/ml). Bound mAbs were detected with horseradish peroxidase-conjugated rabbit anti-mouse IgG secondary Ab (1/1000 dilution) and 4-chloro-1-naphthol substrate (Sigma) (Hong et al., 2006).

2.4. Immunostaining of native chTL1A

2.4.1. Immunohistochemistry in lymphoid tissues

Thymus, caecal tonsils, and bursa of Fabricius tissues were removed from 3-week-old Cobb 500 broilers (Grupo Sada, Zaragoza, Spain) and immediately frozen in liquid nitrogen. Cryostat tissue sections were prepared on glass slides and incubated in proteinase K (Life Technologies) at 27 °C for 2 min. Non-specific protein binding sites were blocked at room temperature for 10 min with 10% normal horse serum in PBS. Tissue sections were incubated with chTL1A mAb-1 (1:50 dilution in PBS) at 4 °C for 18 h, followed by 90 min with FITC-conjugated rabbit anti-mouse IgG secondary Ab (Sigma). Tissue sections were observed with an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan). As negative controls, the procedure was repeated in the absence of chTL1A mAb.

2.4.2. Capture ELISA for determining serum TL1A concentrations

To show the utility of these mAbs in the detection of native chTL1A in chicken serum, an antigen capture ELISA

was developed using the two mAbs specific for chTL1A. The TL1A mAb-1 was used to coat at 0.1 µg in carbonate buffer/well onto 96 well microtitre plates overnight at 4 °C. The plates were blocked and washed as described previously (Lee et al., 2013).

Chicken sera from the control and the necrotic enteritis (NE)-afflicted groups ($n=12/\text{group}$) derived by infecting *Eimeria maxima* and *Clostridium perfringens* (Lee et al., 2013) and collected at 6 days post infection were diluted by 1:2 in PBS-T, 100 µl added to the wells, and incubated for 2 h at room temperature. NE was induced as described (Park et al., 2008). The plates were washed with PBS-T, and 100 µl/well of peroxidase-conjugated TL1A mAb-2 (1 µg/ml) was added and incubated for 30 min followed by development with 3,3',5,5'-tetramethylbenzidine substrate. Optical densities at 450 nm were measured and serum chTL1A concentrations were determined using a standard curve generated with known concentrations of rchTL1A.

2.5. Neutralization effect of chTL1A mAbs on chTL1A functions

2.5.1. Lymphocyte proliferation

Chicken splenocytes ($1 \times 10^6/\text{ml}$) were incubated with medium alone, 1000 ng/ml lipopolysaccharide (LPS) as a positive control, rchTL1A protein between 125 and 1000 ng/ml, or 1000 ng/ml rchTL1A protein pretreated with TL1A or IL8 mAb at the indicated concentrations at 41 °C for 48 h. Cell proliferation was measured using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8, Dojindo Molecular Technologies, Gaithersburg, MD) at OD₄₅₀ as described (Lee et al., 2011a,b).

2.5.2. Nitric oxide (NO) production

HD11 chicken macrophages ($1 \times 10^6/\text{ml}$) were incubated with medium alone as a negative control, 1000 ng/ml LPS (Sigma) or recombinant chicken interferon γ (rchiFN-γ) as a positive control, or the indicated concentrations of rchTL1A protein for 24 h. To assess the inhibition of chTL1A mAb on rchTL1A-stimulated NO production, HD11 cells were incubated with 320 ng/ml rchTL1A protein pre-treated with the indicated dilutions of IL8 or chTL1A mAb. Following incubation, 100 µL of cell culture supernatants were transferred to fresh flat-bottom 96-well plates, mixed with 100 µL of Griess reagent (Sigma), and the plates were incubated for 15 min at room temperature. The optical densities were measured at 540 nm using a microplate reader and nitrite concentrations (µM) were determined using a standard curve generated with known concentrations of sodium nitrite (Lee et al., 2009).

2.5.3. Tumor cell cytotoxicity

LSCC-RP9 chicken tumor cells ($5 \times 10^5 \text{ cells/ml}$, 100 µl), a retrovirus-transformed B cell line (Lee et al., 2009), were cultured in 96-well plates with 100 µl/well of medium alone as a negative control, recombinant chicken natural killer (rchNK)-lysin (500 ng/ml) as a positive control, chTL1A protein (16, 62, 250, 500 ng/ml), chTL1A protein (500 ng/ml) pre-incubated with chIL8 or chTL1A mAb (31,

125, 500, 1000 ng/ml) at 41 °C for 48 h. Commercial chTL1A protein expressed in Yeast (Kingfisher Biotech Inc, MN) was used to detect whether the chTL1A mAbs affect its function against chicken tumor cells. Cell proliferation was measured using WST-8 (Dojindo Molecular Technologies) at OD₄₅₀ as described (Lee et al., 2011a,b).

2.6. Statistical analysis

Each sample was analyzed in quadruplicate. All data were subjected to one-way analysis of variance using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL) and were expressed as mean ± SEM values. The differences of mean values between control and treated sample with different concentration of chTL1A or mAb were analyzed using the t-test, and significant differences were statistically considered at $p < 0.05$.

3. Results and discussion

3.1. Production of mAbs against chTL1A

Two mouse hybridomas (chTL1A-1 and chTL1A-2) secreting mAbs specific for chTL1A protein were identified and cloned. Both chTL1A-1 and chTL1A-2 mAbs were of the IgG1 isotype and recognized rchTL1A protein by Western blot analysis (Fig. 1A). The 32.0 kDa protein band recognized by both mAbs corresponded to the predicted molecular weight of a fusion protein containing the full-length 26.0 kDa chTL1A protein plus the 6 kDa epitope tag encoded by the pET32a vector. The chTL1A mAbs did not react with the chIL-8/pET32a or proteinase K-pretreated chTL1A on Western blots (Fig. 1A), verifying their specificity for the intact chTL1A.

3.2. Immunostaining of native chTL1A by chTL1A mAbs

3.2.1. Immunocytochemical analysis of endogenous chTL1A expression

ChTL1A-secreting cells were found in the thymus, caecal tonsils, and bursa of Fabricius (Fig. 1B). In the thymus, immunostaining cells were clustered in the central medulla (Fig. 1B, 1-1). Most of the chTL1A⁺ thymic cells were elongated, with one long process. In the caecal tonsils, the majority of chTL1A⁺ cells showed polygonal or elongated cell body shapes, and were located in the diffuse lymphoid tissue. ChTL1A⁺ cells were not observed in the germinal centers (Fig. 1B, 2-1). In the bursa, chTL1A⁺ cells appeared round in shape, lacked any kind of cellular processes, and were located in the follicles at the outer portion of the cortex. No chTL1A⁺ cells were identified within the follicle-associated epithelium or in the medulla of the follicles (Fig. 1B, 3-1). In Fig. 1B, 1-2, 2-2, and 3-2 show tissues of thymus, caecal tonsils, and bursa of Fabricius stained with second Ab only and there was no chTL1A⁺ cells identified.

3.2.2. Determination of serum TL1A concentration

In our previous study, chTL1A expression in spleen and intestine was increased in the NE-afflicted chickens compared with the uninfected control broiler chickens (Hong et al., 2012). The results from capture assay using two TL1A

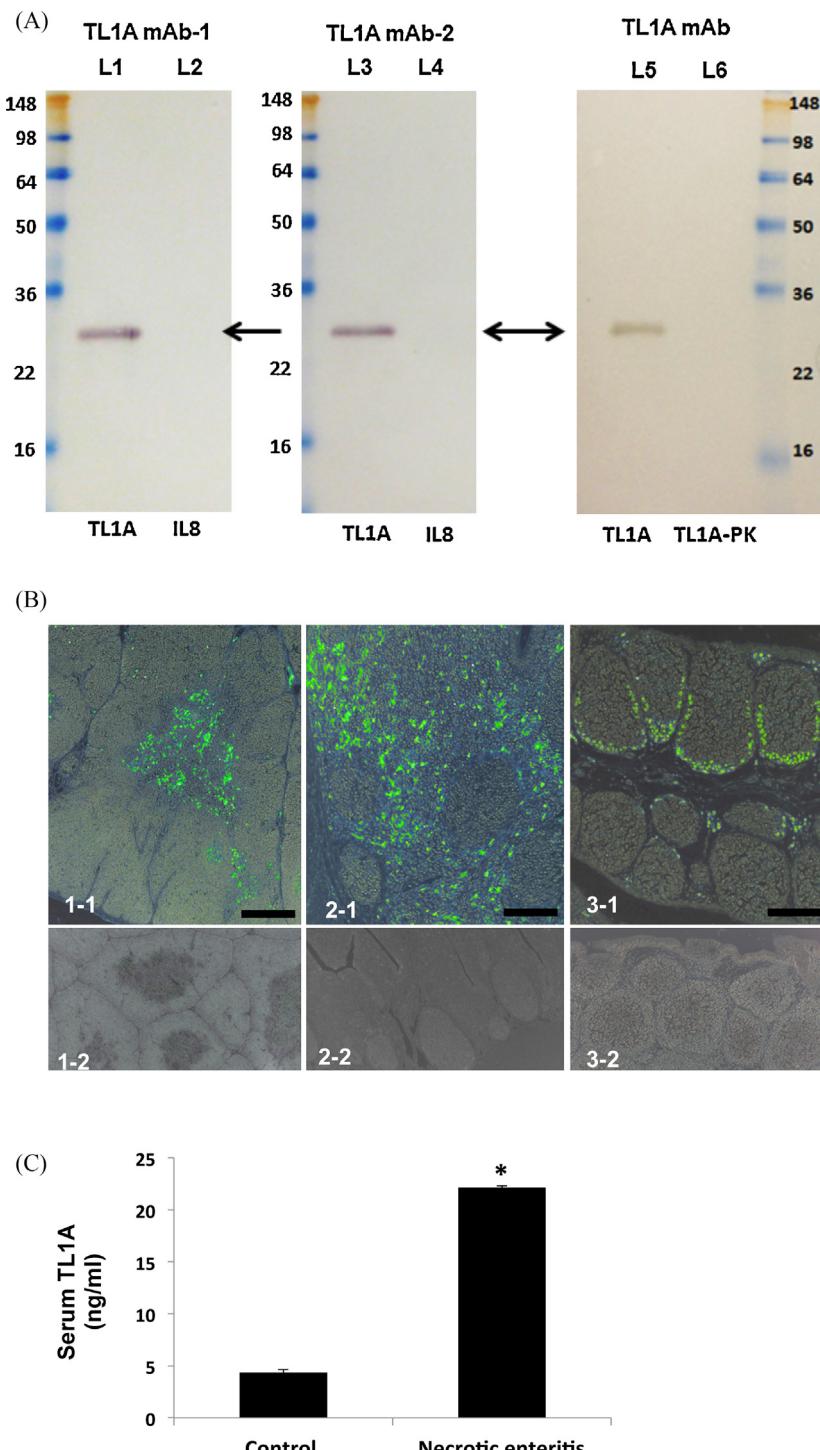
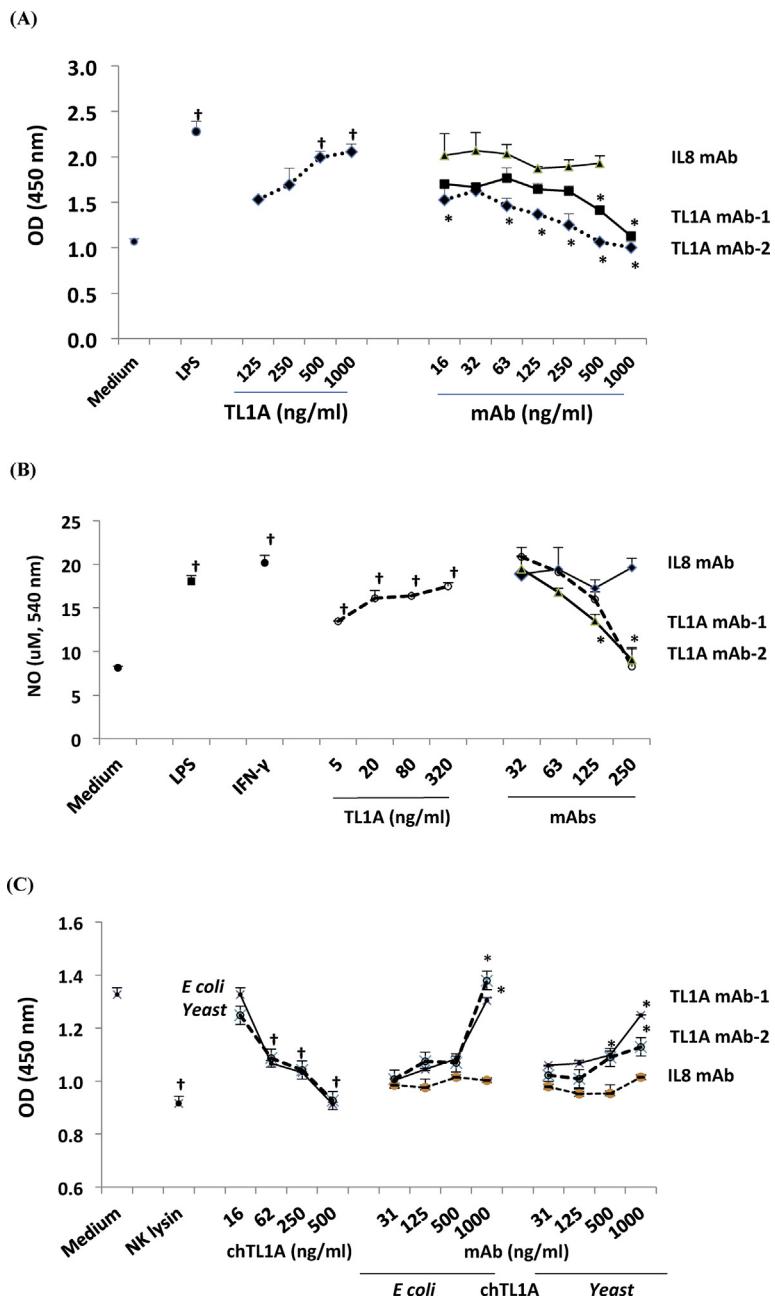


Fig. 1. Western blot analysis and detection of natural chTL1A by chTL1A mAbs.

Purified rchTL1A protein (2 µg/lane (L)—L1, L3, and L5), rchIL8 protein (2 µg/lane—L2 and L4), and the rchTL1A protein (2 µg/lane—L 6) treated with proteinase K at 100 ng/ml were analyzed by Western blot using chTL1A-1 (L1, L2), chTL1A-2 (L3, L4), and chTL1A (mixture of 2 mAbs) mAbs. The position of protein molecular weight markers is indicated on the left or right in kDa.

Thymus (1), cecal tonsil (2), and bursa of Fabricius (3) tissues were snap frozen in liquid nitrogen, and tissue sections were stained with chTL1A mAb-1. Bars = 300 µm (1-1), 150 µm (2-1), and 100 µm (3-1). Fig. 1-2, 2-2, and 3-2 show negative control tissues of thymus, cecal tonsil, and bursa of Fabricius stained with second Ab only.

Serum TL1A protein levels in chickens without and with necrotic enteritis were measured by capture assay using the chTL1A mAbs. The TL1A mAb-1 was used to coat at 0.1 µg in carbonate buffer/well onto 96 well microtitre plates and 100 µl/well of peroxidase-conjugated TL1A mAb-2 (1 µg/ml) was used as a detecting Ab. Each data point represents the mean ± SEM value ($n=12$). * $p<0.001$ compared with the uninfected control according to the *t*-test.

**Fig. 2.** Inhibition of chTL1A bioactivity by chTL1A mAbs.

(A) Chicken splenocytes ($1 \times 10^6/\text{ml}$) were incubated with medium alone as a negative control, 1000 ng/ml LPS as a positive control, rchTL1A protein from 125 to 1000 ng/ml, or 1000 ng/ml rchTL1A pretreated with IL8 or TL1A mAb at the indicated concentrations for 48 h. Cell proliferation was measured at OD₄₅₀ using WST-8.

(B) HD11 chicken macrophages ($1 \times 10^6/\text{ml}$) were incubated with medium alone as a negative control, 1000 ng/ml LPS or rchIFN- γ as a positive control, or the indicated concentrations of rchTL1A protein for 24 h. To assess inhibition of rchTL1A-stimulated NO production, HD11 cells were incubated with 320 ng/ml rchTL1A protein pretreated with the indicated dilutions of IL8, chTL1A-1, or chTL1A-2 mAb. NO levels in cell culture supernatants were measured at OD₅₄₀ using Griess reagent.

(C) LS99-RP9 chicken tumor cells ($5 \times 10^5/\text{ml}$) were cultured with medium alone as a negative control, 500 ng/ml rchNK-lysin as a positive control, the indicated concentrations of rchTL1A protein, or 125 ng/ml rchTL1A pretreated with IL8 or TL1A mAb at 31, 125, 500, or 1000 ng/ml for 24 h. Commercial chTL1A expressed in yeast was compared with the E. coli expressed chTL1A. Cell viability was measured at OD₄₅₀ using WST-8.

In (A), (B), and (C), each data point represents the mean \pm SEM value ($n=3$). †, $p < 0.01$ compared with medium alone; *, $p < 0.05$ compared with rchTL1A alone at 1000 ng/ml (A), 320 ng/ml (B), or 500 ng/ml (C) according to the *t*-test.

mAbs showed that the concentration of serum TL1A protein is significantly ($p < 0.001$) higher in the NE-infected group compared to the control group (Fig. 1C). This finding confirms our previous finding that chTL1A transcript is expressed at higher levels in the NE-afflicted chicken intestine (Park et al., 2007). These results indicate that TL1A mAbs will be useful for measuring serum TL1A levels in chickens with and without enteric diseases, especially in diseases with heightened proinflammatory cytokines in chickens.

3.3. Neutralization effect of chTL1A mAbs on chTL1A protein function

3.3.1. Proliferation of splenocytes

TL1A promotes the expansion of activated T cells (Hou et al., 2005; Haritunians et al., 2010; Jones et al., 2011; Cohavy et al., 2011) and *in vivo* treatment with TL1A Abs has reduced a specific role of TL1A in enhancing T cell proliferation at the sites of tissue inflammation in animal models of autoimmunity (Qin, 2011). In this study, chTL1A mAbs significantly decreased the functional activity of chTL1A on splenocyte proliferation ($p < 0.05$) (Fig. 2A). In contrast, chTL1A pretreated with IL8 mAb did not show any effect on splenocyte proliferation.

3.3.2. NO production by macrophages

Mammalian TNF superfamily cytokines play a critical role in regulating inflammation, in part, through the synthesis of inflammatory mediators such as NO and various cytokines (Haritunians et al., 2010; Migone et al., 2002; Prehn et al., 2007; Meylan et al., 2008). Therefore, we investigated the ability of chTL1A mAbs to neutralize chTL1A-induced NO production by macrophages. As shown in Fig. 2B, chTL1A exhibited a dose-dependent increase in NO levels in cell culture supernatants, compared with cells treated with medium alone. Both chTL1A-1 and chTL1A-2 mAbs inhibited chTL1A-induced NO production in a dose-dependent manner whereas IL8 mAb didn't affect it. Generally, chTL1A-2 mAb was superior to chTL1A-1 mAb in blocking chTL1A-stimulated NO production whereas the chTL1A-1 mAb inhibited chTL1A-stimulated IL-1 β and IL-6 gene expression by macrophages (unpublished observation).

3.3.3. Tumor cell cytotoxicity

Chicken TL1A inhibited the growth of LSCC-RP9 tumor cells in a dose-dependent manner (Park et al., 2008) and decreased the viability of CHO-K1 cells that overexpressed full-length chTNFRs (Takimoto et al., 2008). The TL1A mAbs significantly blocked the cytotoxicity of both *E. coli*- and yeast-expressed chTL1As against LSCC-RP9 tumor cells in a dose-dependent manner (Fig. 2C).

In summary, this paper reports the development and characterization of two new mAbs, chTL1A-1 and chTL1A-2, that detect chTL1A protein by Western blotting, immunocytochemistry, and ELISA capture assay. Furthermore, both mAbs neutralized chTL1A-induced splenocyte proliferation, NO production by chicken macrophages, and blocked the cytotoxic effect of chTL1A protein against LSCC-RP9 tumor cells. These new chTL1A mAbs will be

useful immune reagents for basic and applied research in poultry.

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Conflict of interest

There is no conflicts of interest.

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